Raf Kinase Inhibitory Protein (RKIP): Functional Pleiotropy in the Mammalian Brain

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Abstract

In 1984, a cytosolic protein was isolated from bovine brain and coined phosphatidylethanolamine binding protein (PEBP) to describe its phospholipid-binding potential. Its cellular function remained elusive for more than a decade until it was discovered that PEBP had the ability to suppress the Raf1-mitogen activated protein kinase (MAPK) pathway, earning it the new name of Raf1 kinase inhibitory protein (RKIP). This milestone discovery has paved the way for numerous studies that have now extended the reach of RKIP's function to other signaling cascades, within the context of various physiological and pathophysiological systems. This review will summarize our current knowledge of the neurophysiological roles of RKIP in the mammalian brain, including its function in the circadian clock and synaptic plasticity. It will also discuss evidence for an involvement of RKIP and its derived neuropeptide, hippocampal cholinergic neurostimulating peptide (HCNP), in neural development and differentiation. Implications in certain pathologies such as Alzheimer's disease and brain cancer will be highlighted. By chronicling the diverse functions of RKIP in the brain, we hope that this review will serve as a timely resource that ignites future studies on this versatile, multifaceted protein in the nervous system.

Keywords

Alzheimer's disease; cancer; circadian rhythms; HCNP; long-term depression; neural differentiation; PEBP1; RKIP

I. INTRODUCTION: HISTORY OF RKIP

The RKIP research field as we know it today had its humble origins from a study three decades earlier describing the biochemical isolation and characterization of a soluble basic protein expressed in the bovine brain.¹ Ligand binding studies revealed that this protein could bind in a specific manner to phosphatidylethanolamine (PE), a class of phospholipids that is localized to the cytoplasmic leaflet of the plasma membrane.² As a result, the protein was designated phosphatidylethanolamine-binding protein 1 (PEBP1), which remains the official name of the encoding gene. Progress in understanding the physiological functions of PEBP1 was modest. Then, in 1992, Ojika et al. purified a novel 11-residue peptide from the soluble fraction of neonatal rat hippocampi, and called it hippocampal cholinergic

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neurostimulating peptide (HCNP) because of its ability to stimulate acetylcholine synthesis in cultures of medial septal nuclei, a region of the brain that receives inputs from the hippocampus.³ It would be another three years before the genes that encode rat and human HCNP were cloned, and recognized as homologs of bovine *pebp1*.⁴ The discovery of HCNP was, in some respects, a major step toward ascribing a physiologic activity to this mysterious gene in the mammalian brain.

The breakthrough in the function of this gene came in 1999 when Yeung et al. identified a novel inhibitor of mitogen-activated protein kinase (MAPK) signaling.⁵ PEBP1 now earned a second, and what would become a more widely used, name of Raf1 kinase inhibitory protein (RKIP), due to its ability to inhibit Raf1 kinase. The following decade witnessed an emergence of new reports describing RKIP as a regulator of multiple signaling pathways including and beyond the MAPK cascade. At the same time, there was a growing number of clinical studies that implicated loss of RKIP expression in metastatic cancers, and molecular studies to understand how RKIP might work to influence cancer cell behavior.⁶ Although the momentum has shifted toward elucidating the role of RKIP in cancer, there is no doubt continued interest in the physiological relevance of RKIP in the brain, the organ of its original identification. This review will begin by discussing RKIP's molecular functions with respect to major signaling pathways. A brief description of the regional expression profile of RKIP in the mammalian brain will ensue, followed by a lengthy discussion of RKIP's role in circadian rhythms, synaptic long-term depression (LTD), neural development and differentiation, stress and depression, Alzheimer's disease, and brain cancer.

II. RKIP: DIVERSITY OF CELLULAR AND MOLECULAR FUNCTIONS

In what has become a seminal study on RKIP's cellular function, Yeung et al. used the yeast two-hybrid screen to identify a novel interacting partner of Raf1, the first kinase to be activated within the Ras-Raf1-MEK-ERK signal transduction cassette.^{5,7} Inhibition of endogenous RKIP enhanced ERK (extracellular signal-regulated kinase) activation and downstream AP-1-dependent transcription, whereas overexpression of RKIP inhibited ERK and AP-1 activation, and cellular transformation induced by an oncogenic Raf1 (but not other oncogenes such as *v-fos* or *v-src*). The authors further showed that RKIP, by binding to either Raf1 or MEK (MAPK kinase), competitively inhibited the association between these two kinases, and prevented Raf1-mediated phosphorylation of MEK.^{5,8} These data establish RKIP as the first, and only, physiological inhibitor of Raf1 signaling and have profound implications for numerous biological processes in which the intensity, kinetics, and/or duration of ERK signaling are critical to defining the response.⁹

Subsequent biochemical studies by Yeung et al. revealed that, despite acting as a competitive inhibitor of Raf1-MEK association, RKIP bound to a site on Raf1 that was distinct from the MEK interaction domain.⁸ In addition, the MEK binding site on RKIP encompassed residues 27–77, whereas the interaction with Raf1 required multiple sites including a core site between residues 77–108.⁸ Binding of RKIP to either Raf1 or MEK was sufficient to suppress ERK signaling. Corbit et al. later showed that protein kinase C (PKC) activation could trigger the dissociation of RKIP and Raf1, and thus derepression of ERK signaling. Residue (Ser- or Thr-) 153 of RKIP was identified as the phosphorylation site for certain

isoforms of PKC, and mutation of this site resulted in constitutive suppression of ERK activity.¹⁰ By identifying RKIP as a substrate of PKC phosphorylation, Corbit et al. provided a molecular link between the PKC and ERK signaling pathways.¹⁰

The work of Lorenz et al. was perhaps one of the first hints that RKIP may orchestrate multiple cellular signaling events beyond simply the Raf1-MAPK pathway.¹¹ In that study, the authors showed that RKIP could interact physically with G protein-coupled receptor kinase 2 (GRK2) and inhibit its ability to phosphorylate various G protein-coupled receptors (GPCRs).^{11,12} PKC-mediated phosphorylation of residue 153 on RKIP was required for its ability to inhibit GRK2. Overexpression of wild-type RKIP enhanced receptor signaling and decreased receptor internalization, whereas overexpression of phospho-deficient RKIP had no effect on either parameter, and knockdown of endogenous RKIP suppressed receptor-mediated signaling. Through their investigations, Lorenz et al. revealed that RKIP can switch from Raf1 to GRK2 binding following PKC-dependent phosphorylation, and can thereby simultaneously enhance signaling of two distinct pathways.¹¹

Two other major signaling pathways that are modulated by RKIP are nuclear factor-kappa B (NF- κ B) and glycogen synthase kinase 3 beta (GSK3 β).^{13,14} NF- κ B is a transcription factor that is maintained in an inactive form in the cytoplasm through association with inhibitory kappaB (I κ B). A number of kinases directly and indirectly trigger the phosphorylation, and consequent degradation, of I κ B, resulting in the release of NF- κ B and its translocation into the nucleus. Yeung et al. demonstrated that RKIP could physically interact with four kinases required for NF- κ B activation, and block the phosphorylation of I κ B.¹⁵ Analogous to its effects on Raf1 signaling, overexpression of RKIP inhibited both basal and cytokine-induced NF- κ B-mediated transcription. Later studies revealed that RKIP might also modulate NF- κ B signaling through interactions with components that are acting upstream of I κ B kinases.¹⁶

In a departure from RKIP's recognized role as a signaling inhibitor, Al-Mulla et al. recently discovered that RKIP served as an activator of GSK3 β signaling.¹⁷ RKIP physically associated with GSK3 β , and overexpression of RKIP suppressed the phosphorylation of GSK3 β at residue 390, which would otherwise lead to GSK3 β inactivation. Knockdown of RKIP had the opposite effect by promoting phosphorylation at this inhibitory site (a target site for p38 kinase), and also led to stabilization of GSK3 β protein (Fig. 1).

RKIP's participation in these, and potentially other, signaling pathways puts it in a position where it can have a profound impact on various neurophysiological processes (Table 1). It is worth reiterating that, prior to the emergence of studies embedding RKIP in known signaling cascades, any hint of RKIP's potential function in the brain was limited to insights on HCNP, its derived peptide. In the remainder of this review, we will discuss in detail the studies that have aimed to establish the functional consequences of RKIP, and HCNP, expression in the mammalian brain.

III. PEBP1/RKIP: GENOMIC AND PROTEIN STRUCTURE, SUBCELLULAR DISTRIBUTION, AND REGIONAL EXPRESSION IN THE BRAIN

The *pebp1* gene is composed of four exons on chromosome 12 of the human and rat genome (or chromosome 5 in mice). The open reading frame of full-length RKIP (187 amino acids) spans all four exons, where exon 1 contains the entire coding sequence of HCNP (rat and mouse HCNP: Ala²-Ala-Asp-Ile-Ser-Gln-Trp-Ala-Gly-Pro-Leu¹²), corresponding to residues 2–12 of the RKIP protein. HCNP is believed to be generated from its precursor protein through enzymatic cleavage of the N-terminus.¹⁸ There is some recent evidence to indicate that HCNP, and potentially RKIP itself, is secreted under certain conditions, although how this occurs and through which receptors the secreted peptide acts are outstanding questions.^{19,20} Within the cell, both the work of Bernier et al. and the subsequent elucidation of the crystal structure of RKIP suggest strongly that RKIP is associated with the inner leaflet of the plasma membrane, where it is ideally situated to transduce signals from the membrane to the cytoplasm.^{2,21} On the other hand, immunohistochemical studies have revealed that RKIP may be expressed in multiple subcellular compartments, depending on the cell type or condition: RKIP is localized predominantly in the cytoplasm and at the plasma membrane, with occasional expression detected within the nucleus.^{22–24} The function of nuclear RKIP is unclear, although Eves et al. have shown that RKIP may associate with centrosomes and kinetochores to regulate the spindle checkpoint of mitotic cells.²⁵

A comprehensive analysis of *pebp1* gene expression in the murine brain was performed by Theroux et al. using the β -geo reporter.²⁶ In that study, a gene trap in intron 1 of the murine *pebp1* gene resulted in the integration of the β -geo gene cassette, encoding a chimeric protein composed of the enzymes β -galactosidase and neomycin phosphotransferase, downstream of the endogenous *pebp1* promoter. X-gal staining in adult mouse brains revealed widespread *pebp1* expression with a broad range of intensities (from very weak to strong). Strongest staining was observed in limbic structures, including virtually all nuclei of the hypothalamus and amygdala, all CA fields of the hippocampus, the pyramidal cell layer and granular layer of the dentate gyrus, the nucleus accumbens, and anterior olfactory nuclei. Within the cerebellum, *pebp1* expression was restricted to the Purkinje cell layer. The expression profile of *pebp1* in the brain suggests that it might regulate processes as diverse as: motivation, pleasure and addiction; learning and memory, including spatial and motor; olfaction; aggression, fear, and anxiety; neuroendocrine-related homeostasis; and regulation of circadian cycles, sexual arousal, thirst, and hunger. In fact, in the original Theroux et al. study, it was reported that RKIP-null mice exhibited an olfactory deficit that became more severe as the animals aged.²⁶

IV. RKIP AND ENTRAINMENT OF THE CIRCADIAN CLOCK

The molecular characterization of RKIP as a Raf1 kinase inhibitory protein capable of repressing mitogen-activated protein kinase (MAPK) signaling prompted the initial speculation by our group that RKIP might play a role in circadian clock regulation. In mammals, the central circadian pacemaker resides in a region of the anterior hypothalamus

known as the suprachiasmatic nucleus (SCN).²⁷ The molecular machinery that drives endogenous, near-24 h rhythms is expressed in cellular oscillators of the SCN and elsewhere in the periphery, and consists of interacting transcriptional-translational feedback loops (TTFLs) of core clock genes and their gene products. Circadian rhythms of behavior and physiology are synchronized (or entrained) to the day-night cycle as a result of direct resetting of the retinally innervated SCN clock by photic (light) stimuli. Numerous studies have shown that light information is conveyed to the molecular clockwork through glutamate and pituitary adenylate cyclase activating peptide (PACAP)-induced activation of ERK signaling within the SCN, and subsequent activation of cAMP response element-binding protein (CREB)-dependent transcription of the clock genes, *Period1* (*Per1*) and *Period2* (*Per2*).^{28–30} Changes in PER expression in the SCN are believed to be the ultimate underlying basis of clock resetting. Moreover, pharmacological inhibition of ERK signaling using U0126, a selective inhibitor of MEK1 and MEK2, is sufficient to abrogate the clock-resetting effects of light, indicating the critical function of ERK activation in clock entrainment.³¹ One interesting point that is often overlooked is that light-induced ERK activation in the SCN follows a strict temporal pattern that is marked by rapid induction within minutes of light onset and return to basal activity 1 h following light onset, regardless of the duration of light exposure.³² These results suggest that mechanisms exist that limit the duration of ERK activation, which might play an important role in determining the final biological response. Additional kinases have also been implicated in clock entrainment. One of these is the family of PKC proteins, which have been shown to phosphorylate RKIP at residue 153 and abolish its inhibition of Raf1.^{33–35}

Our initial biochemical studies helped to set the stage for a possible role of RKIP in light-induced entrainment of the SCN circadian clock.³⁶ RKIP protein was detected at moderate to high levels throughout the entire murine SCN. Co-immunoprecipitation experiments indicated a physical interaction between RKIP and Raf1 in the SCN under basal conditions, and partial dissociation of this complex shortly following exposure to a resetting light pulse. This weakened affinity of RKIP for Raf1 coincided with a light-triggered increase in RKIP Thr-153 phosphorylation. Interestingly, this phosphorylation event is critical for clock resetting at both the behavioral and molecular levels: transgenic mice that overexpressed the non-phosphorylatable RKIP point mutant, RKIP T153V (containing a Thr-153→Val-153 conversion), in the SCN and forebrain exhibited a profound reduction in light-induced ERK activation in the SCN and behavioral phase shifts.

Using *rkip*-nullizygous mice generated by Theroux et al., we showed that RKIP acts as a physiological negative modulator of the clock-resetting effects of light.^{26,36} In the absence of *rkip* expression, mice exhibited exaggerated (larger) responses to the clock-delaying and -advancing effects of brief light exposure in the early and late night, respectively. Furthermore, in an experimental model that mimics air travel from Los Angeles to London, *rkip*-deficient mice required significantly fewer days to readjust their clock to an 8 h advance in the light-dark schedule, suggesting an accelerated recovery from jet lag. However, RKIP does not seem to play an essential role in maintaining endogenous rhythms, since *rkip*-deficient mice exhibited normal free-running behavioral rhythms under conditions of constant darkness with a circadian period that was not significantly different from wild-type

controls. This lack of effect on core timing mechanisms was reinforced by the observation that PER1 and PER2 protein rhythms were unaltered in the SCN of *rkip*-null mice.

At the molecular level, loss of RKIP expression had a marked effect on light-evoked ERK activity within the SCN.³⁶ Although the initial activation of ERK appeared not to be different between wild-type and *rkip*-deficient mice, reaching the same level of activation 15 min after light onset, ERK activity was sustained for a prolonged length of time in the knockout strain. This effect was observed when light was delivered in both the early and late night, when the larger phase delays and advances were evident in this strain. Prolonged ERK activity in *rkip*-deficient SCN also correlated with enhanced transcriptional activation of the *Per1* and *c-Fos* genes, as well as greater numbers of PER1- and PER2-immunoreactive cells, following photic stimulation.

In aggregate, the evidence indicates that RKIP negatively regulates light-induced entrainment of the SCN clock.³⁶ The duration of light-evoked ERK activity in the SCN, which is modulated by RKIP, may be causal to this entrainment phenotype, or it may be only coincidental. At present, it is unclear the degree to which the kinetics of ERK activity, rather than simply the amplitude of induction, dictates both the qualitative and quantitative properties of the SCN's response to light. In extreme cases such as cellular differentiation, transient versus sustained ERK activation is decoded differently by the cell to produce a specific and distinct response.³⁷ Additionally, the study by Antoun et al. did not assess the potential impact of *rkip* ablation on GRK2, NF- κ B, or GSK2 β signaling in the SCN, and their contribution to the entrainment phenotype of the knockout mice.³⁶ GRK2 signaling is particularly germane to this debate, since the SCN utilizes a number of GPCRs, including the receptor for PACAP, to regulate photic resetting.³⁸ Nevertheless, as the first study to implicate RKIP in circadian rhythms, the work of Antoun et al.³⁶ provides a basis to further investigate RKIP-regulated signaling events in the SCN.

V. RKIP AND LONG-TERM DEPRESSION

Another recent study on the role of RKIP in cerebellar long-term depression (LTD) also arose from its strategic position at the intersection of PKC and MAPK signaling.³⁹ LTD is an activity-dependent process that selectively weakens neuronal synapses in a long-lasting fashion. In conjunction with long-term potentiation (LTP), or the persistent increase in synaptic strength, LTD is a mechanism that is used in different brain regions to influence synaptic plasticity, the basis for many forms of learning and memory. Within the cerebellum, LTD is induced by brief co-stimulation of parallel fibers and climbing fibers, both of which project to cerebellar Purkinje cells.⁴⁰ The short-term stimulation of these fibers triggers the activation of second messenger pathways in Purkinje cells, including increase in cytosolic calcium levels and production of inositol triphosphate (IP₃), which ultimately leads to LTD. However, these second messengers return to baseline levels within seconds after synaptic activity is terminated, whereas LTD persists for minutes to hours. Tanaka and Augustine had previously discovered that these transient second messenger signals are translated into sustained kinase activity through a positive feedback loop that involves the reciprocal activation of PKC and MAPK.⁴¹ Importantly, this PKC-MAPK positive feedback loop is

required for cerebellar LTD. Other molecular factors are likely required within the feedback loop to directly link PKC and MAPK.

The recent work of Yamamoto et al. provided evidence that RKIP is the pivotal PKC effector that feeds into and regulates the MAPK pathway during cerebellar LTD.³⁹ RKIP is expressed robustly in the Purkinje cell layer of the cerebellum, primarily localizing to the somata and dendrites of Purkinje cells, the site where cerebellar LTD occurs. RKIP expression was also observed in distal dendrites and dendritic spines, indicating a potential role in synaptic function that might contribute to LTD. To test whether RKIP is functionally involved in cerebellar LTD, the authors examined the effects of disrupting PKC-mediated phosphorylation of RKIP using the point mutants RKIP-TV (Thr-153→Val-153 conversion) and RKIP-TA (Thr-153→Ala-153 conversion), thus constitutively inhibiting Raf1-MEK signaling, in Purkinje cells. Following the introduction of purified RKIP-WT (wild type), RKIP-TV, and RKIP-TA into Purkinje cells by patch pipette, the authors quantified the strength of LTD using a paired electrical stimulation protocol. The RKIP-TV and RKIP-TA mutants caused a significant reduction in the amount of LTD when compared to RKIP-WT, indicating that phosphorylation of RKIP is important for LTD formation. Notably, this decrease in LTD was not observed when another RKIP mutant, RKIP-TV-delBD, carrying the T153V mutation along with deletion of the binding site for Raf1 and MEK (amino acids 27–108) was used, suggesting that the dissociation of phospho-RKIP from Raf1 and MEK is critical for cerebellar LTD.^{8,39}

A series of experiments confirmed that RKIP worked downstream of PKC and upstream of MAPK activation to induce cerebellar LTD.³⁹ LTD can be elicited by direct activation of PKC or MAPK using the phorbol ester, TPA, or by photically uncaging a constitutively active form of MEK1 (MEK-CA), respectively. Uncaging of MEK-CA in Purkinje cells in which RKIP-TV was co-introduced caused LTD induction to the same extent as in RKIP-TV-delBD controls, signifying that RKIP is dispensable for LTD once MAPK is activated. On the other hand, TPA-triggered LTD was abolished in Purkinje cells containing RKIP-TV, indicating that PKC's effects on LTD requires the actions of RKIP. Collectively, these data establish PKC→RKIP→MAPK as the sequence of events leading to cerebellar LTD.

To complement the electrophysiological evidence, the authors examined the physical associations between RKIP and its functional partners.³⁹ Immunoprecipitation confirmed the interaction between RKIP and Raf1/MEK in the cerebellum under basal conditions. Co-stimulation with potassium and glutamate, which induces LTD, disrupted the RKIP/Raf1/MEK complex. Importantly, this dissociation was prevented in the presence of a PKC inhibitor. The results indicate that RKIP interacts with Raf1 and MEK under basal conditions, and dissociates from these proteins in a PKC-dependent manner following stimulation leading to LTD induction.

The study by Yamamoto et al. provides the first physiological evidence to implicate RKIP in PKC-mediated activation of the MAPK pathway in the murine cerebellum, within the context of LTD induction.³⁹ Previous work indicated that activated MAPK goes on to phosphorylate phospholipase A2 (PLA2), which produces arachidonic acid (AA) to further activate PKC and complete the positive feedback loop.^{41–43} Similar to the study by Antoun

et al., the work of Yamamoto et al. focused solely on the PKC-MAPK signaling axis, and did not address the potential contribution of RKIP to other signaling pathways that participate in cerebellar LTD induction. For instance, GRK2 has been shown to desensitize the metabotropic glutamate receptor, mGluR1, a key player in cerebellar LTD.^{44,45} Future studies should address whether RKIP-dependent modulation of GRK2 activity is working in concert with the PKC-MAPK positive feedback mechanism to drive LTD induction.

VI. RKIP, HCNP, AND NEURAL DEVELOPMENT

Since the discovery of HCNP in the rat hippocampus by Ojika et al., a number of studies have implicated HCNP and its precursor protein, RKIP, in neuronal differentiation.³ The original studies showed that HCNP stimulated the development and differentiation of cholinergic neurons in cultures of rat medial septal nuclei, by enhancing the activity of choline acetyltransferase (ChAT) and thus promoting acetylcholine synthesis.^{3,46} These medial septal cholinergic neurons project back to the hippocampus to form the septohippocampal cholinergic pathway, whose dysfunction has been linked to cognitive impairments, particularly those associated with aging.⁴⁷ HCNP's ability to enhance ChAT expression specifically in the septal medial nucleus, and promote cholinergic phenotype development, was later confirmed *in vivo* using transgenic mice that overexpressed HCNP in the murine forebrain and hippocampus.⁴⁸

In humans, the expression of HCNP in the hippocampus is highest during the perinatal and early postnatal period, gradually declines thereafter until it reaches trough levels at age 10 years, and is maintained in this low state throughout adulthood.⁴⁹ Older patients (60–80 years of age) tend to exhibit higher levels of HCNP compared to young adults.⁴⁹ In mice, there are similar age-related changes in HCNP-related antigens in the hippocampus: peak levels are observed at two months, followed by a gradual decline from two to five months, and a moderate increase by eight months.⁵⁰ This inverse relationship between age and HCNP expression, excluding old age, suggests that HCNP and its precursor may be involved in neural development during childhood. Interestingly, in a senescence-accelerated mouse strain, HCNP-related antigens gradually accumulate in the hippocampus from birth to eight months without a decrement phase from two to five months as observed in control mice.⁵⁰ The increase in HCNP in senescence-accelerated mice from three to five months correlated with memory deterioration that is evident in this strain.⁵¹

Additional evidence for a role of HCNP and its precursor in neural development comes from a number of *in vitro* studies. Proteomic analysis of conditioned medium from cultured adult rat hippocampal progenitor cells (AHPs) revealed that RKIP is secreted by AHPs.⁵² One potential function of RKIP may be to promote differentiation of cells down the neuronal lineage rather than the astroglial lineage. Retinoic acid (RA)-induced neuronal differentiation of SH-SY5Y human neuroblastoma cells was enhanced by overexpression of RKIP, and suppressed by silencing of RKIP.⁵³ RKIP-silenced cells adopted an astroglial-like morphology and had enhanced expression of the glial marker, glial fibrillary acidic protein (GFAP).⁵³ Even in cells that were not treated with RA, overexpression of RKIP alone was sufficient to induce neuronal differentiation to a limited extent.⁵³ Interestingly, long-term ethanol exposure of RA-treated SH-SY5Y cells, which impairs neuronal differentiation, also

triggered a significant downregulation of RKIP and protein kinase C γ (PKC γ) protein expression as well as suppressed brain derived neurotrophic factor (BDNF)-induced ERK activation.⁵⁴ Similar findings were observed in rat AHPs, where endogenous RKIP expression closely correlated with differentiation into neurons and oligodendrocytes, but not GFAP-positive astrocytes.⁵⁵ Consistent with this, overexpression of RKIP promoted neuronal and oligodendrocyte differentiation, whereas RKIP silencing promoted differentiation into astrocytes.⁵⁵

Using an *in vivo* stroke model in neonatal rat pups, Toyoda et al. provided evidence for a role of RKIP in the differentiation of hippocampal neural precursor cells following hypoxia-ischemia (HI).⁵⁶ RKIP expression was detectable in a proportion of actively proliferating cells in the hippocampi of HI brains. More than half of these RKIP-immunoreactive cells expressed the oligodendrocyte lineage marker CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase), whereas none colocalized with the astrocyte marker GFAP. The data suggest that RKIP suppresses differentiation into astroglial lineages while promoting oligodendrocyte differentiation.

Collectively, these studies provide a body of evidence to support a role of RKIP/HCNP in neural cell fate determination. The data suggest that RKIP/HCNP promotes differentiation toward neuronal and oligodendrocytic lineages, and suppresses astroglial differentiation. What remains unclear from these studies is the relative contributions of HCNP versus its precursor protein in neural differentiation. If the effects are primarily accounted for by HCNP itself, what is the mechanistic basis of its activity? If it is the precursor protein that is eliciting these effects, which downstream signaling pathways are responsible? Beyond differentiation, does RKIP regulate other aspects of neural development such as cell cycle control or apoptosis? Clearly, these early studies on RKIP/HCNP have opened new and exciting avenues of research aimed at clarifying RKIP's role in neural development.

VII. RKIP IN STRESS AND DEPRESSION

There is some evidence using animal models that links the levels of RKIP to stress and depression, two conditions that are associated with suppression of neurogenesis (the generation of new neurons) in the hippocampus.⁵⁷ Chronic restraint-induced stress downregulated the expression of RKIP in the rat brain; a parallel reduction in RKIP was observed in rat hippocampal cell lines stimulated with the synthetic glucocorticoid (stress hormone), dexamethasone.⁵⁸ Conversely, administration of the antidepressants, fluoxetine or venlafaxine, to adult rats enhanced the expression of RKIP by twofold in the hippocampus.⁵⁹ Whether or not the increase in RKIP expression is related to the stimulatory effects of these antidepressants on AHP proliferation is unknown.⁵⁹ The role of RKIP in depressive-like behavior was examined using transgenic mice overexpressing RKIP in the fore-brain.⁶⁰ In both the forced swim test and tail suspension test, RKIP transgenic mice exhibited enhanced depressive-like behavior that was apparent in aged subjects (30 weeks old) but not a younger cohort (12 weeks old).⁶⁰ The cognitive impact of RKIP overexpression was restricted to depression, as behaviors associated with anxiety and spatial learning and memory were not affected in these animals.⁶⁰ Although data from the transgenic mouse model seem to be at odds with implications arising from the effects of antidepressant

treatment on RKIP expression, one cannot exclude the possibility that endogenous RKIP might have a vastly different effect from its transgenic counterpart due to cell- and/or region-specific expression as well as the physiological range of expression levels. It remains to be seen how disruptions in endogenous RKIP expression might influence depressive-like behavior.

VIII. RKIP AND ASSOCIATION WITH ALZHEIMER'S DISEASE

A number of studies have addressed the potential contribution of RKIP to Alzheimer's disease (AD), the most common form of dementia diagnosed in the aging population. AD is characterized by a suite of neuropathological changes, such as accumulation of β -amyloid plaques and neurofibrillary tangles in the brains of AD patients, in addition to progressive impairment in cognitive functions including learning and memory.⁶¹ Several hypotheses have been proposed to provide a causative basis for the disease.⁶² One of these, the cholinergic hypothesis, proposes that the cognitive impairments in AD patients are the result of a deficiency in acetylcholine synthesis, which, as discussed earlier, is promoted by HCNP.⁶³ Consistent with a potential role of HCNP in AD, levels of *pebp1* mRNA are suppressed in the CA1 region of the hippocampi of clinically late-onset AD patients relative to non-demented controls.⁶⁴ Given previous evidence that shows that AD pathologies are localized in the CA1 region and there is loss of cholinergic fibers innervating the hippocampus, this study suggests that reduced expression of HCNP may contribute to the pathogenesis of AD. In comparison, earlier work showed that the cerebrospinal fluid (CSF) of some early-onset, but not late-onset, AD patients exhibits high levels of HCNP relative to non-AD patients.⁶⁵ Compared to AD patients exhibiting normal levels of HCNP in the CSF, those with higher levels showed a greater degree of cerebral cortex degeneration.⁶⁵ Additionally, HCNP/RKIP has been shown to accumulate in Hirano bodies, which are intracellular aggregates found in nerve cells of the elderly and patients with certain forms of neurodegenerative diseases.^{66,67} HCNP/RKIP-immunoreactive Hirano bodies are much more prevalent in the hippocampal CA1 field of AD patients than in age-matched non-AD individuals.⁶⁶ Although these clinical studies suggest an association between changes in RKIP expression and AD, their functional relationship is unclear at present.

One study using an animal model of AD supports the association between RKIP and AD. The Tg2576 transgenic mouse model, which overexpresses the "Swedish" mutant form of the human amyloid precursor protein (APP) gene, exhibits a lower abundance of RKIP protein (but not transcript) selectively in the hippocampus.^{68,69} RKIP levels in Tg2576 mice decrease significantly with age starting at 11 months, whereas non-transgenic mice do not exhibit this age-related decline. The decline in RKIP abundance at 11 months coincides with an increase in amyloid-beta ($A\beta$) plaque accumulation in the brains of Tg2576 mice. RKIP immunoreactivity is detected at the periphery of $A\beta$ plaques, and in select astrocytes surrounding these plaques.⁶⁸ The authors concluded that the accumulation of $A\beta$ plaques may affect the expression of PEBP1/RKIP protein, which further contributes to the pathogenesis of AD.⁶⁸

The cumulative evidence implicates RKIP as a potential factor in AD. One obvious question that arises is whether changes in RKIP expression are contributing to the dysfunction in key

signaling pathways observed in AD brains. For instance, increased GSK3 activity has been linked to AD, and may contribute to many of the hallmark features of AD.⁷⁰ In addition, A β -induced neuroinflammation in AD brains may involve the NF- κ B pathway.⁷¹ Future studies will hopefully define the role of RKIP in these cellular pathways as they relate to AD pathogenesis.

IX. RKIP AND ASSOCIATION WITH BRAIN CANCER

There is overwhelming clinical and nonclinical research evidence establishing RKIP as a cancer-critical gene.⁶ Different types of cancers, including prostate, pancreatic, colorectal, breast, and others, are associated with loss of RKIP expression, especially during the metastatic (invasive) stage.^{23,72–74} Not surprisingly, RKIP is often referred to as a metastasis suppressor gene, and loss of its expression is generally correlated with a poor prognosis.⁷⁵ In terms of cancers of the brain, four studies to date have analyzed RKIP expression in brain tumor samples obtained from human patients. In the earliest study, RKIP protein levels were profoundly suppressed in higher-grade (malignant) astrocytomas compared to non-neoplastic samples and lower-grade astrocytomas.⁷⁶ An independent clinical study corroborated these findings in primary glioma tissues compared to adjacent normal glial tissues, and human glioma cell lines versus control human glial cells.⁷⁷ A third clinical study showed that overall survival was best for high-grade gliomas that are positive for RKIP and negative for STAT3 (signal transducer and activator of transcription 3) expression.⁷⁸ However, in another report, Martinho et al. found that only a small proportion (i.e., approximately 10%) of gliomas from human patients are associated with loss of RKIP expression.⁷⁹ Evidence from *in vitro* experiments using human glioma cell lines is consistent with a role of RKIP as a metastasis suppressor, since RKIP inhibition promotes cell migration, whereas its overexpression suppresses glioma cell invasion.^{77,79} On the other hand, an *in vivo* assay using cultured human glioma cells implanted into chick chorioallantoic membrane did not find evidence for an effect of RKIP suppression on cellular proliferation and angiogenesis.⁷⁹

The cumulative evidence from this handful of studies supports a potential involvement of RKIP in human gliomas. One might ask what the mechanisms are that underlie the loss or downregulation of RKIP expression in malignant gliomas. Mechanisms including gene methylation and transcriptional repression by the epithelial-mesenchymal transition (EMT)-promoting factor, SNAIL, have been implicated in *rkip* gene regulation in other types of cancers.^{80,81} Another important issue is to identify the molecular basis of RKIP's effects on tumor malignancy, invasion, and metastasis. Are the signaling pathways that are known to be influenced by RKIP involved? Does loss of RKIP expression affect the expression of specific genes that are important for tumor cell invasion and migration, and interactions between the cancer cell and its microenvironment? These questions and more await future investigations.

X. CONCLUDING REMARKS

As we mark the 30th anniversary of the discovery of RKIP, we are left with a sense of awe at the remarkable progress that has been made to understand the function of this most versatile

of proteins. Clearly, RKIP is not simply a Raf kinase inhibitory protein, nor just a phosphatidylethanolamine-binding protein, or even merely a precursor of hippocampal cholinergic neurostimulating peptide. The breadth of RKIP's involvement in various signaling pathways is extensive, as is its physiological impact in multiple tissues, not the least of which is the brain. From neural development to the control of circadian rhythms and cerebellar LTD, from aging and Alzheimer's disease to brain cancer, RKIP has established itself as a central player in many processes that underlie human health and disease. Looking forward to the next decade, we can expect to uncover even more functions of this protein, and to elaborate on the already extensive map of RKIP-dependent signaling in the mammalian brain.

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ABBREVIATIONS

AA	arachidonic acid
AD	Alzheimer's disease
AHP	adult hippocampal progenitor cell
AP-1	activator protein 1
BDNF	brain-derived neurotrophic factor
CA	constitutive active
cAMP	cyclic AMP
ChAT	choline acetyltransferase
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CREB	cAMP response element-binding protein
CSF	cerebrospinal fluid
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptor
GRK2	G protein-coupled receptor kinase 2
GSK3β	glycogen synthase kinase 3 beta

HCNP	hippocampal cholinergic neurostimulating peptide
HI	hypoxia-ischemia
IκB	inhibitory kappaB
LTD	long-term depression
IP₃	inositol triphosphate
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
mGluR1	metabotropic glutamate receptor 1
NF-κB	nuclear factor kappaB
PACAP	pituitary adenylate cyclase activating peptide
PE	phosphatidylethanolamine
PEBP1	phosphatidylethanolamine binding protein 1
Per1	Period1
Per2	Period2
PKC	protein kinase C
PLA₂	phospholipase A2
RA	retinoic acid
RKIP	Raf1 kinase inhibitory protein
SCN	suprachiasmatic nucleus
STAT3	signal transducer and activator of transcription 3
TTFL	transcriptional-translational feedback loop
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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FIG. 1.

Diagram of the role of RKIP in various signaling pathways. RKIP inhibits the ERK/MAPK pathway by preventing Raf1 from associating with MEK, and on phosphorylation via PKC inhibits activation of GRK2. It also prevents the phosphorylation-induced inhibition of GSK3 β by p38 mitogen activated protein kinase. Finally, RKIP exerts an inhibitory effect on the NF κ B signaling pathway by interacting with kinases responsible for the phosphorylation of I κ B and subsequent dissociation from NF κ B. This event leads to I κ B degradation and relief of inhibition of NF κ B.

TABLE 1

Summary of the roles of RKIP in the brain

Function/model system	Description	Refs.
Signaling	MAPK Inhibitor of MAPK pathway. Unphosphorylated RKIP binds to Raf1, inhibiting its association with MEK	5,7,8
	GRK2 PKC-dependent inhibitor of GRK2. PKC-phosphorylated RKIP binds to and inhibits activity of GRK2	10–12
	NFκB Inhibitor of NFκB. Inhibits phosphorylation of Inhibitory κB through interaction with upstream kinases	15,16
	GSK3β Activator of GSK3β by associating with its inhibitory site	17
Circadian rhythms	Negative regulator of light-induced entrainment of the circadian clock through inhibition of MAPK signaling	36
Cerebellar LTD	PKC-dependent dissociation of phospho-RKIP from Raf1 and MEK is essential for cerebellar LTD induction	39,40
Neural development	Highest <i>rkip</i> expression levels at early postnatal and old age Promotes differentiation to neuronal and oligodendrocytic lineages Suppresses astroglial fate	49–51, 53–55
Stress	Downregulation of <i>rkip</i> expression after stress and glucocorticoid stimulation	57,58
Depression	Loss of <i>rkip</i> correlates with depressive-like behavior in older mice Antidepressant administration enhances expression	59,60
Alzheimer's disease	Deregulation of hippocampal <i>rkip</i> transcript/protein expression in AD	64–69
Brain cancer	Loss of <i>rkip</i> expression is associated with gliomas	76–79